

Genetic diversity among isolates of *Fusarium oxysporum* f.sp. *canariensis*

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Fusarium oxysporum f.sp. *canariensis* causes vascular wilt disease of *Phoenix canariensis*, the Canary Island date palm. Seventy-two isolates of this fungus were obtained from diverse geographic locations including France, Japan, Italy, the Canary Islands, and California, Florida and Nevada, USA. The isolates were tested for vegetative compatibility and for similarities based on mitochondrial DNA (mtDNA), single-copy sequences and repetitive DNA (pEY10) polymorphisms. Seventy-one percent of the isolates belonged to a single vegetative compatibility group (VCG 0240), and four closely related mitochondrial RFLP patterns were found. A subset of the isolates was further tested for single-copy RFLPs and repetitive DNA fingerprints. Only four single-copy RFLP haplotypes were found among 25 representative isolates of *F. oxysporum* f.sp. *canariensis* tested, using nine polymorphic single-locus probe/enzyme combinations. Finally, 32 different pEY10 DNA fingerprints were found out of 57 isolates examined. Overall the results indicate that *F. oxysporum* f.sp. *canariensis* is a single lineage with a low to moderate level of genetic diversity.

Keywords: DNA fingerprinting, *Fusarium*, genetic diversity, palm wilt, *Phoenix canariensis*, vegetative compatibility

Introduction

The soilborne fungus *Fusarium oxysporum* contains many host-specialized, pathogenic forms or *formae speciales* that cause vascular wilt and/or root rot disease in particular plant species. *Fusarium oxysporum* f.sp. *canariensis* (Mercier & Louvet, 1973) causes a severe vascular disease known as Fusarium wilt on the Canary Island date palm (*Phoenix canariensis*). Fusarium wilt is currently threatening these ornamental palms in landscapes and nurseries in many parts of the world. Molecular genetic studies indicate that there are different patterns of diversity exhibited by various *formae speciales* of *F. oxysporum* (Kistler, 1997). Some have few multilocus haplotypes based on single-locus RFLP data, have similar or identical mitochondrial RFLP haplotypes, and/or belong to a single vegetative compatibility group (VCG). This simple pattern of diversity is found in *F. oxysporum* f.spp. *albedinis* (Tantaoui & Fernandez, 1993; Tantaoui *et al.*, 1996; Fernandez *et al.*, 1997) and *conglutinans* (Bosland & Williams, 1987; Kistler *et al.*, 1987), and is consistent with a population structure resulting from the clonal

expansion of a single, successful pathogenic genotype. Other *formae speciales* have patterns of diversity that also are consistent with clonality, but are characterized by two or more distinct lineages within a *forma specialis*, as reflected in multiple VCGs, several diverse mitochondrial haplotypes, and relatively larger numbers of multilocus haplotypes defined by RFLPs or randomly amplified polymorphic DNAs (RAPDs). This pattern is exemplified by *F. oxysporum* f.spp. *cubense* (Bentley *et al.*, 1995; Koenig *et al.*, 1997; O'Donnell *et al.*, 1998), *melonis* (Jacobson & Gordon, 1988, 1990) and *lycopersici* (Elias & Schneider, 1991; Elias *et al.*, 1993). Distance measurements between lineages within these *formae speciales* indicate that they may be very closely related or quite distant. This research is the first to examine the relationships of isolates within *F. oxysporum* f.sp. *canariensis* with respect to vegetative compatibility and molecular characteristics. It is still to be determined whether this *forma specialis* consists of a single lineage, like *F. oxysporum* f.spp. *albedinis* and *conglutinans*, or whether it is made up of multiple lineages like *F. oxysporum* f.spp. *cubense*, *melonis* and *lycopersici*. If *F. oxysporum* f.sp. *canariensis* is a single lineage, then detection by a single diagnostic method such as that developed by Plyler *et al.* (1999) would be practical. This information could also influence strategies for deployment of disease resistance and disease management.

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Table 1 *Fusarium oxysporum* f.sp. *canariensis* isolates and outgroups listed by geographic origin, vegetative compatibility to VCG 0240 testers, mitochondrial haplotype, RFLP multilocus haplotype and pEY10 fingerprint

Isolates	Geographic origin*	Vegetatively compatible	Mitochondrial haplotype	RFLP multilocus haplotype†	pEY10 fingerprint
87-Guil2 ^a	Tenerife, CI	No	A	H111112111 E111111111	5
87-Guil7 ^a	Tenerife, CI	No	A	H111112111 E111111111	4
87-Lago3 ^a	Tenerife, CI	Yes	A	H111112111 E111111111	5
84-01a ^a	Cannes, France	ND	A	ND	9
84-01b ^a	Cannes, France	No	A	H211112111 E111111111	9
84-02b ^a	Cannes, France	No	A	ND	9
84-P1 ^a	Cannes, France	Yes	A	H211112111 E111111111	9
84-P2 ^a	Cannes, France	No	A	H211112111 E111111111	9
70-MerclV ^a	Provence, France	No	A	H111111111 E111111111	6
94-868 ^b	Manatee Co., FL	ND	A	ND	ND
94-275 ^b	Manatee Co., FL	Yes	A	ND	18
94-865 ^b	Manatee Co., FL	Yes	A	ND	ND
94-867 ^b	Manatee Co., FL	Yes	A	ND	ND
94-869 ^b	Manatee Co., FL	Yes	A	ND	ND
94-871 ^b	Manatee Co., FL	Yes	A	ND	18
94-872 ^b	Manatee Co., FL	Yes	A	ND	ND
94-873 ^b	Manatee Co., FL	Yes	A	ND	ND
94-2418 ^b	Manatee Co., FL	Yes	A	ND	ND
95-914 ^b	Manatee Co., FL	Yes	A	ND	18
95-1724 ^b	Manatee Co., FL	Yes	A	ND	18
95-1725 ^b	Manatee Co., FL	Yes	A	ND	ND
95-1726 ^b	Manatee Co., FL	Yes	A	ND	18
95-1714 ^b	Dade Co., FL	Yes	A	H111111111 E111111111	14
95-2121 ^b	Palm Beach Co., FL	Yes	A	ND	24
96-148 ^b	Lee Co., FL	ND	A	ND	ND
84-Cala ^a	California	No	A	H111111111 E111111111	ND
95-2122 ^b	California	Yes	A	ND	19
95-2123 ^b	California	No	A	ND	ND
96-1672 ^b	California	No	A	ND	19
96-1673 ^b	California	No	A	ND	19
96-1613 ^b	Nevada	Yes	A	H311112111 E111111111	28
95-F0505F ^b	California	Yes	B	H111111111 E111121111	3
95-F051F ^b	California	Yes	B	H111111111 E111121111	1
95-F052F ^b	California	Yes	B	ND	20
95-F053F ^b	California	Yes	B	ND	21
96-08 ^b	California	Yes	B	H111111111 E111111111	2
96-09 ^b	California	Yes	B	ND	ND
96-13 ^b	California	Yes	B	ND	2
96-14 ^b	California	Yes	B	ND	2
95-513 ^b	Manatee Co., FL	Yes	B	ND	13
95-514B ^b	Manatee Co., FL	Yes	B	ND	ND
95-514C ^b	Manatee Co., FL	No	B	ND	13
95-913 ^b	Manatee Co., FL	Yes	B	H211112111 E111111111	13
95-1000 ^b	Lake Co., FL	Yes	B	ND	13
95-1001 ^b	Lake Co., FL	No	B	ND	13

Table 1 continued

Isolates	Geographic origin*	Vegetatively compatible	Mitochondrial haplotype	RFLP multilocus haplotype†	pEY10 fingerprint
95-1002 ^b	Lake Co., FL	Yes	B	ND	13
95-1003 ^b	Lake Co., FL	Yes	B	ND	13
95-1004 ^b	Lake Co., FL	Yes	B	H211112111 E111111111	13
95-1005 ^b	Lake Co., FL	No	B	ND	26
95-1006 ^b	Lake Co., FL	No	B	ND	13
95-1007 ^b	Lake Co., FL	No	B	ND	13
95-861 ^b	Hillsborough Co., FL	No	B	H211112111 E111111111	11
96-1416 ^b	Hillsborough Co., FL	Yes	B	ND	27
95-1488 ^b	Pinellas Co., FL	Yes	B	H111111111 E111111111	12
95-1772 ^b	Broward Co., FL	Yes	B	H111111111 E111111111	15
95-2269 ^b	Palm Beach Co., FL	Yes	B	H111111111 E111111111	16
96-540 ^b	Palm Beach Co., FL	Yes	B	ND	29
96-847 ^b	Sarasota Co., FL	No	B	ND	30
84-104 ^a	Kagoshima, Japan	Yes	B	H111111111 E111111111	7
84-305 ^a	Kagoshima, Japan	No	B	H111111111 E111111111	7
Sicily 1 ^c	Sicily, Italy	Yes	B	H211112111 E111111111	10
Sicily 2 ^c	Sicily, Italy	No	B	H211112111 E111111111	10
94-556 ^b	Manatee Co., FL	Yes	C	ND	17
94-2140 ^b	Sarasota Co., FL	Yes	C	H111111111 E111111111	17
95-456 ^b	Lee Co., FL	Yes	C	ND	31
95-1008 ^b	Lake Co., FL	Yes	C	ND	22
95-1009 ^b	Lake Co., FL	Yes	C	ND	23
95-1731 ^b	Broward Co., FL	Yes	C	ND	32
96-1733 ^b	Dade Co., FL	Yes	C	ND	ND
96-1889 ^b	Volusia Co., FL	Yes	C	ND	ND
84-207 ^b	Kagoshima, Japan	No	D	H111111111 E111111111	8
94-841 ^b	Sarasota Co., FL	Yes	D	ND	25
Outgroups					
F9127	South Africa	ND	E	H211111111 E111112111	33
<i>F. o. cubense</i>					
Dem1/EQ	Ecuador	ND	F*	H222213112	ND
<i>F. o. elaeidis</i>				E2122 1211	
699	Wisconsin, USA	ND	G	H412223111	34
<i>F. o. raphani</i>				E311233221	

F. oxysporum f.sp. canariensis isolates from:

^athe collection of Diana Fernandez, IRD, Montpellier, France;

^bthe collection of Gary W. Simone, Florida Extension Plant Disease Clinic, Gainesville, FL, USA;

^cQuirico Migheli, Università di Torino, Italy.

*The abbreviation Co. represents the county in Florida from which the isolates were obtained.

†RFLP fingerprints were generated with *Hind*III (H) or with *Eco*RV (E) using the polymorphic probes 31, 91 and 106, represented in this order within the haplotype: H or E (91, –, –, –, 31, 106, –, –, –).

‡Two isolates of *F. oxysporum f.sp. elaeidis* from Ecuador were used for analysis. EQ was tested with the mitochondrial probe but not with single-copy probes. Dem1 was tested with single-copy probes but not the mitochondrial probe.

Materials and methods

Fungal isolates

Seventy-two isolates of *F. oxysporum* f.sp. *canariensis* were used to measure genetic diversity (Table 1). These included 13 isolates (three from the Canary Islands, six from France, three from Japan and one from California, USA) that were confirmed to be *F. oxysporum* f.sp. *canariensis* via pathogenicity tests (J. Louvet, INRA, Dijon, France, personal communication). The remaining isolates used in this study came from tissues of *Phoenix canariensis* showing symptoms, which were sent to the Florida Extension Plant Disease Clinic in Gainesville from locations in Florida, California, and Nevada, or to the Università di Torino from Sicily. These isolates were identified by primers that detected the authentic palm pathogens and by mitochondrial RFLP profiles that were similar to the original pathogen strains (Plyler *et al.*, 1999). *Fusarium oxysporum* f.sp. *cubense* isolate F9127, f.sp. *raphani* isolate 699, f.sp. *elaeidis* isolate EQ and f.sp. *elaeidis* isolate Dem1 were used as outgroups. All DNA was extracted according to the methods discussed previously, and single spores of the isolates were stored in 50% glycerol at -80°C (Plyler *et al.*, 1999).

Vegetative compatibility determination

Single-spore isolates of *F. oxysporum* f.sp. *canariensis* were removed from -80°C and placed onto PDA. After 2–5 days' incubation at room temperature, a 1 cm^2 block of mycelium was removed from the margin of the actively growing culture and placed onto PDA containing 30 g L^{-1} potassium chlorate. Chlorate-resistant sectors would appear from 5 to 9 days as an area of wild-type growth (Puhalla, 1985).

A 1 cm^2 block of mycelium from the chlorate-resistant mutants was placed onto basal medium, which consisted of 30 g sucrose, 1 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g KCl, 10 mg FeSO_4 , 20 g laboratory grade agar, 0.2 mL trace element solution (5 g citric acid, 5 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 0.25 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 50 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 50 mg H_3BO_3 , 50 mg $\text{NaMoO}_4 \cdot \text{H}_2\text{O}$ per 95 mL water), amended with 2 g NaNO_3 in 1 L water to make minimal medium. Mutants unable to use nitrate appeared as a layer of thin mycelial growth (Correll *et al.*, 1987).

To identify hypoxanthine nonutilizing mutants (nitM), a 1 cm^2 block of mycelium from the minimal medium was placed on hypoxanthine media containing 0.2 g L^{-1} hypoxanthine added to the basal medium (Correll *et al.*, 1987). Two mutants, derived from isolates 94-871 and 95-F051F, were identified by their inability to use hypoxanthine as a sole nitrogen source and appeared as a thin layer of mycelial growth on this medium. These two mutants were used as nitM testers for vegetative compatibility pairings.

To test for vegetative compatibility, a 1 cm^2 block of mycelium from one of the nitM testers was placed in

the centre of a plate containing minimal medium. A 1 cm^2 block of chlorate-resistant mutants or nitrate-nonutilizing mutants derived from three other isolates were placed adjacent to the testers and allowed to grow together. Heterokaryon formation, which appeared as an area of dense, wild-type growth, occurred between 3 and 14 days. All isolates were tested against both hypoxanthine mutants (94-871 and 95-F051F). If an isolate formed a heterokaryon with either or both of these mutants, it was considered to be in VCG 0240, as assigned by the VCG numbering coordinator (Katan, 1999). If one of the nit mutants in the plate paired with an adjacent nit mutant, these were considered vegetatively compatible as well. If one of the two adjacent nit mutants had paired with the tester, all three isolates were considered to be within VCG 0240. Isolates that formed heterokaryons were, by definition, in the same vegetative compatibility group.

Mitochondrial haplotypes

DNA was extracted according to the methods described previously (Plyler, 1997). Approximately $1\text{ }\mu\text{g}$ of total DNA from all of the *F. oxysporum* f.sp. *canariensis* isolates and from 20 other isolates of *F. oxysporum* was digested with *Hae*III, loaded onto 0.7% agarose gels and run between 19 and 22 h at 33 V. The gels were prepared for Southern blotting on a Nytran nylon membrane (Schleicher and Schuell, Keene, NH, USA) using capillary transfer methods essentially as described previously (Sambrook *et al.*, 1989). The membrane was exposed to UV light (254 nm) to immobilize the DNA.

For the hybridization probe, mitochondrial DNA (15 μg) was extracted from *F. oxysporum* f.sp. *cubense* isolate 3S1 (Koenig *et al.*, 1997). The DNA was digested with *Hind*III, labelled, hybridized to the membrane at 65°C overnight, and detected on autoradiography film according to the manufacturer's protocol (Dupont NEN 'Renaissance', E. I. Du Pont de Nemours and Co., Boston, MA, USA).

Single-copy RFLPs

A subset of 25 isolates were chosen for RFLP analysis so that each geographic location of the isolates was represented (Table 1). DNA was extracted as previously described from single-spore cultures of each isolate. Approximately $1\text{ }\mu\text{g}$ of total DNA was digested with either *Hind*III, *Xba*I, *Eco*RI, *Dra*I or *Eco*RV for 7–24 h at 37°C . The digested DNA was run on 0.7% agarose gels for 19–22 h at 31–34 V. The DNA was transferred by Southern blotting to Zeta Probe nylon membranes with 0.4 M NaOH. The DNA was fixed by heating at 80°C for 30 min.

Seventeen clones out of a plasmid library of total DNA from isolate 95-913 were screened against the digested DNAs for polymorphisms. The clones were labelled by nick translation with ^{32}P (Gibco BRL Life

Technologies, Gaithersburg, MD, USA). Hybridization of the clones to the membrane was made at 65°C according to the methods described previously (Plyler, 1997). After hybridization, the membranes were exposed to autoradiography film at -80°C for 1–2 days depending on the strength of the signal.

DNA fingerprinting

Approximately 1 µg of total DNA from each of the *F. oxysporum f.sp. canariensis* isolates and from the outgroup isolates was digested with *HindIII*, loaded onto 0.7% agarose gels and run at 33 V for 18–21 h. The gels were prepared for Southern blotting using the alkaline capillary transfer methods previously mentioned for single-copy RFLPs.

The fingerprinting probe used in this study, pEY10, was obtained from a genomic library of DNA from *F. oxysporum f.sp. conglutinans* (Kistler *et al.*, 1991) and corresponds to an open reading frame with a predicted amino-acid sequence having 51% identity to the

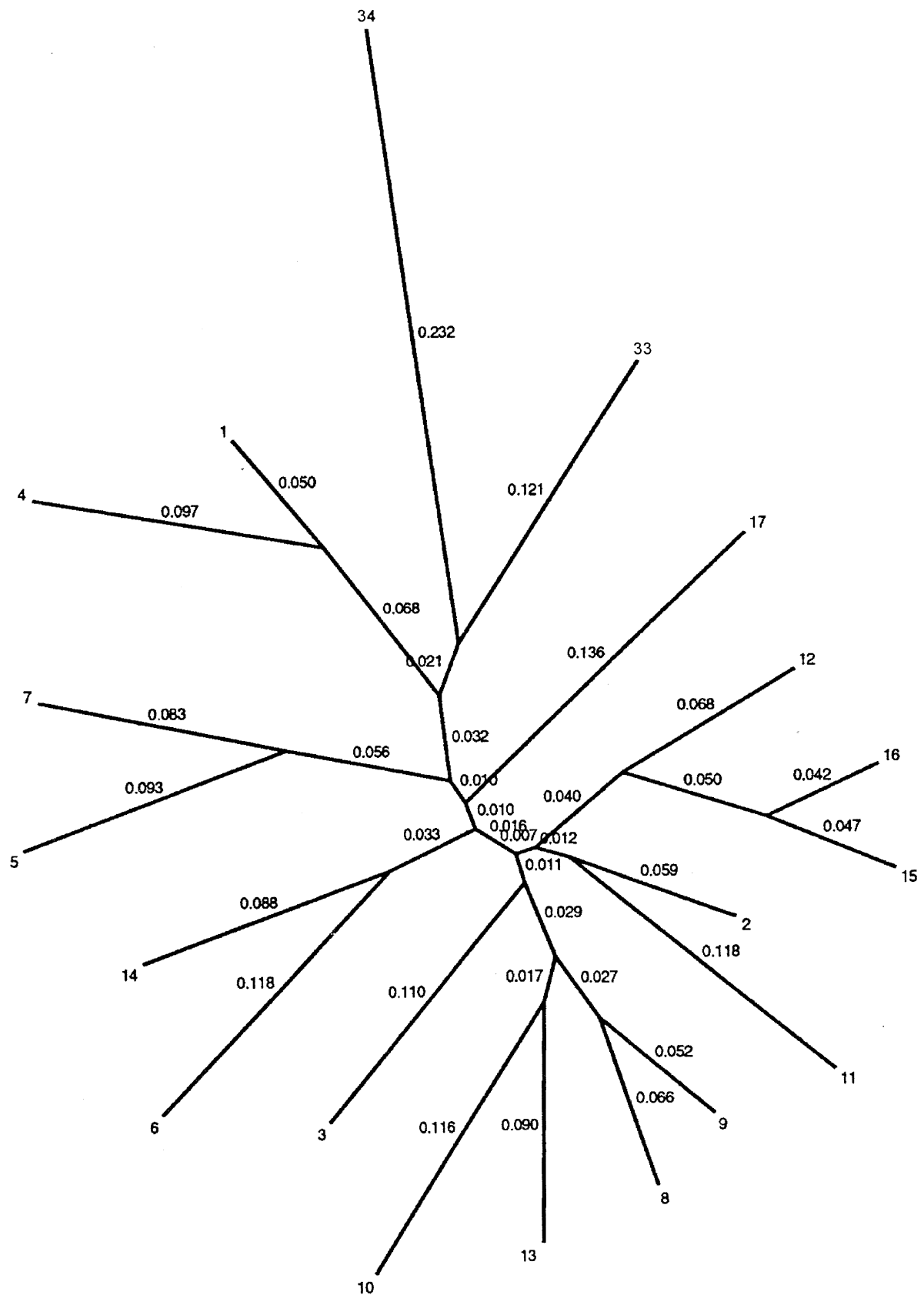
putative transposase from *Magnaporthe grisea* repetitive element *Pot3* (R.E. Pettway and H.C. Kistler, personal communication). DNA from pEY10 was labelled by nick translation (Gibco BRL). Hybridization of the probe to the membrane and detection of the signal was performed as described in the methods for single-copy RFLPs.

Data analysis

The pEY10 fingerprints were obtained for 57 *F. oxysporum f.sp. canariensis* isolates, 23 of which were also tested with the single-copy RFLP probes. A matrix of polymorphic restriction sites was created for these 23 isolates, with the exception of 84-Cala and 96-1613, and entered into the matrix (Fig. 1). A migration position on the gel was determined for each band detected using this probe. The presence or absence of a band at each migration position was scored 1 or 0, respectively. Genetic distance was determined using the heuristic search option in PAUP*4.0d64 (Swofford, 1998) for the pEY10 fingerprint data (Fig. 2). *Fusarium*

Band ↓	Fingerprint number																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	33	34	
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	
2	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	
3	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	
4	0	0	1	0	0	1	0	0	0	1	0	1	0	1	0	0	1	0	0	
5	0	0	0	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	
6	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	
8	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	
9	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
11	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	1	
12	0	1	1	1	0	0	1	0	0	1	1	1	1	1	1	1	1	0	0	
13	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	
14	0	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	
15	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	
16	0	0	1	1	0	1	0	1	1	1	0	1	1	0	1	1	0	0	0	
17	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	
18	1	0	1	1	1	0	1	1	1	1	1	0	1	1	0	0	1	0	1	
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
20	0	0	0	0	0	1	1	0	1	1	1	0	0	1	0	1	1	0	1	
21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	
22	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	
23	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
24	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	
25	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	
26	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	
27	1	1	1	0	1	0	1	0	1	1	0	1	1	0	1	1	1	1	1	
28	0	1	1	0	0	1	0	1	1	1	1	1	1	0	1	1	0	1	0	
29	0	0	0	0	0	0	1	0	0	0	1	1	0	0	1	1	1	0	0	
30	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
31	0	0	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0	1	
32	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
33	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	
34	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	

Figure 1 Matrix of band positions for pEY10 fingerprint. 1, presence of band; 0, absence of band.



oxysporum f.sp. *cubense* and *raphani* were used as outgroups.

Results

VCG determination

Approximately 71% of isolates (49 of 69) formed heterokaryons with 94-871 and/or 95-F051F nitM testers, or with a nit mutant that had paired with one of the testers. Nit tester mutants of 94-871 or 95-F051F did not pair with each other, but could be linked by the nit mutant of isolate 95-513 that paired with both of them. Isolates that paired included isolates from every country represented in this study. All isolates that formed heterokaryons with the testers or that could be linked to the testers were considered a single vegetative compatibility group (VCG 0240). Among *F. oxysporum* f.sp. *canariensis* isolates unable to pair with either VCG 0240 tester, isolates 95-514C and 95-1005 formed heterokaryons with each other, as did isolates 96-1672 and 87-Guil2.

The remaining isolates did not form heterokaryons with any other isolate of *F. oxysporum* f.sp. *canariensis*. These included three isolates from California, five from Florida (three from Lake County, one from Hillsborough County and one from Sarasota County), two of the three isolates from Japan, one of the two isolates from Sicily, four isolates from France, and one of the three isolates from the Canary Islands. Nit mutants of these isolates were not further characterized.

Mitochondrial haplotypes

Four mitochondrial haplotypes were observed by hybridization of mitochondrial DNA to DNA from isolates of *F. oxysporum* f.sp. *canariensis* digested with *Hae*III (Fig. 3). The haplotypes were very similar and appeared to represent all four combinations of two length mutations. One of the mutations appeared as an insertion/deletion (indel) in the largest *Hae*III fragment (7 or 5.5 kb) of the *F. oxysporum* f.sp. *canariensis* mitochondrial genome. The other indel resulted in the presence or absence of an approximately 750 bp band after *Hae*III digestion.

The two most commonly occurring haplotypes (A, B) each corresponded to 43.1% of the isolates (Table 1). The less common haplotypes (C, D) represent profiles for 11 and 2.8% of the isolates, respectively (Table 1).

Mitochondrial haplotype did not strictly correlate with the geographic origins of the isolates. All haplotypes were found among the isolates from Florida. Only the two most common haplotypes were found among isolates from California. The three isolates from Japan had haplotypes B and D, whereas all the isolates from

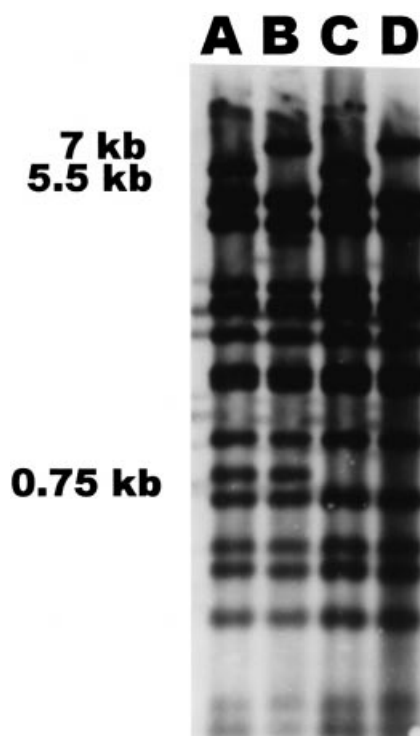


Figure 3 Mitochondrial haplotypes generated from total *F. oxysporum* f.sp. *canariensis* DNA digested with *Hae*III and probed with mitochondrial DNA from *F. oxysporum* f.sp. *cubense* isolate 3S1. Letters A–D correspond to the mitochondrial haplotype designation shown in Table 1.

France and the Canary Islands had mitochondrial haplotype A. The 20 isolates of *F. oxysporum* tested from hosts other than *P. canariensis* all produced banding patterns different from those of the four haplotypes found for *F. oxysporum* f.sp. *canariensis* (data not shown).

Single-copy RFLPs

Polymorphisms were detected using 9 of 17 probes in DNA of *F. oxysporum* isolates, including three strains from other hosts. Of these nine, only three enzyme/probe combinations (*Hind*III/probe 91, *Hind*III/probe 106 and *Eco*RV/probe 31) revealed polymorphisms among DNAs from *F. oxysporum* f.sp. *canariensis* isolates. Using *Eco*RV/probe 31, only two haplotypes were observed within *F. oxysporum* f.sp. *canariensis* (Table 1). With *Hind*III, probes 91 and 106 each produced polymorphisms and together resulted in four haplotypes (Table 1).

The most common haplotype with *Eco*RV (111111111) was found in combination with all four

Figure 2 Unrooted dendrogram of pEY10 fingerprint data. Branches show the genetic distance as determined by PAUP*4-0d64. Numbers at the ends of branches represent the pEY10 fingerprint designation given in Table 1.

mitochondrial haplotypes. The second single-locus haplotype (111121111) was found in only one mitochondrial haplotype and for only two *F. oxysporum* f.sp. *canariensis* isolates, 95-F050F and 95-F051F (Table 1). The most commonly occurring single-locus haplotype with *Hind*III was found for isolates that contained all four mitochondrial haplotypes and were from a diversity of locations. The isolates from the Canary Islands produced a distinctive single-locus haplotype.

All multilocus single-copy haplotypes for the three isolates of *F. oxysporum* from other hosts were different from those of *F. oxysporum* f.sp. *canariensis* and from each other. As anticipated from previous work (O'Donnell *et al.*, 1998; Plyler *et al.*, 1999), *F. oxysporum* f.sp. *cubense* isolate F9127 had a haplotype very similar to that of the *F. oxysporum* f.sp. *canariensis* isolates. Using *Hind*III in combination with the nine probes, isolate F9127 shared alleles with *F. oxysporum* f.sp. *canariensis* at all loci, but had a unique combination of alleles. The isolates Dem1 and 699 produced haplotypes that were different for almost every probe/enzyme combination. These outgroups rarely shared alleles with the *F. oxysporum* f.sp. *canariensis* isolates.

DNA fingerprinting

DNA fingerprints for 57 *F. oxysporum* f.sp. *canariensis* isolates were obtained with the probe pEY10. Thirty-two fingerprint patterns were found for the 57 isolates. The fingerprint patterns appear to align with the geographic origins of the isolates in some cases. For instance, all isolates from Cannes, France have the same fingerprint. On the other hand, isolates from the same geographic location did not always share the same DNA fingerprint. For example, isolate 84-207 from Kagoshima, Japan had a different DNA fingerprint from isolates 84-305 and 84-104 also from Kagoshima. Isolate 84-207 also had a different mitochondrial haplotype from that of the latter isolates.

The ability to form heterokaryons did not correlate with pEY10 fingerprint patterns. Most isolates with identical DNA fingerprint patterns also formed heterokaryons with either nitM 94-871 or nitM 95-F051 tester isolates. However, all but six isolates with unique pEY10 fingerprint patterns were also vegetatively compatible with the nitM testers.

While isolates that shared pEY10 DNA fingerprint patterns also shared single-copy multilocus RFLP haplotypes (Table 1), isolates with different pEY10 fingerprints also sometimes shared multilocus RFLP haplotypes. For example, among the eight isolates having RFLP haplotype 21112111 created by *Hind*III, four different DNA fingerprinting patterns were discerned. The isolates of *F. oxysporum* from other hosts displayed pEY10 fingerprinting patterns unlike those for *F. oxysporum* f.sp. *canariensis* isolates.

The fingerprints from a group of isolates from Florida (95-913, 95-1000 to 95-1007, 95-513 and 95-514C) are similar to those of isolates from Sicily (Sicily 1 and Sicily

2), France (84-01a, 84-01b, 84-P1, 84-P2 and 84-02b), and Japan (84-207). Many of the isolates from Florida did not share the same fingerprint. Fourteen individual DNA fingerprints were detected in the 32 isolates of *F. oxysporum* f.sp. *canariensis* from Florida.

When DNA fingerprinting data were used for distance analysis, dendrograms showed that outgroups *F. oxysporum* f.sp. *raphani* (699) and *F. oxysporum* f.sp. *cubense* (F9127) were distinct from *F. oxysporum* f.sp. *canariensis* isolates. The *F. oxysporum* f.sp. *canariensis* isolates were separated only by a small genetic distance.

Discussion

Several lines of evidence suggest that *F. oxysporum* f.sp. *canariensis* consists of a single clonal lineage with a moderate level of genetic diversification within that lineage. The first line of evidence, vegetative compatibility testing, indicates that there is a single major VCG (0240) within this *forma specialis*, with 71% of the isolates tested belonging to this VCG. Isolates that did not belong to VCG 0240 were nevertheless very similar to, or even indistinguishable from, authentic VCG 0240 isolates based on other genetic criteria (Table 1). However, it is likely that other VCGs exist in *F. oxysporum* f.sp. *canariensis*, as suggested by the fact that isolates 95-514C and 95-1005, as well as isolates 96-1672 and 87-Guil2, formed heterokaryons with each other but not with the nitM testers of VCG 0240. These VCGs might have arisen by mutation at one or more loci for vegetative compatibility.

Comparisons of the VCG information with the other genetic markers used in this study also revealed that all *F. oxysporum* f.sp. *canariensis* isolates were similar. Only four closely related mitochondrial haplotypes were described for the 72 isolates of *F. oxysporum* f.sp. *canariensis* examined. Isolates from each of the four mitochondrial haplotypes were found to belong to VCG 0240. In other *formae speciales* of *F. oxysporum*, mtDNA haplotypes also show strong correlation with vegetative compatibility groups (Jacobson & Gordon, 1990; Gordon & Okamoto, 1992a, b; Tantaoui *et al.*, 1996). The mtDNA profiles for *F. oxysporum* f.sp. *canariensis* contained all four combinations of two length mutations. These combinations of alleles could have resulted from mitochondrial recombination between isolates, or alternatively could have arisen through independent mutations occurring at high frequency at particular sites on the mitochondrial chromosome.

Single-copy haplotype data also support a moderate level of genetic diversity with the probes tested in this study. Upon examination of the single-copy RFLP haplotypes, it was found that typing with the enzyme/probe combinations *Hind*III/91 and *Hind*III/106 can be used to distinguish isolates from the Canary Islands. In fact, the single-copy haplotype of the Canary Island isolates (11112111) differed from all other single-copy haplotypes by only a single step (Fig. 4). No other

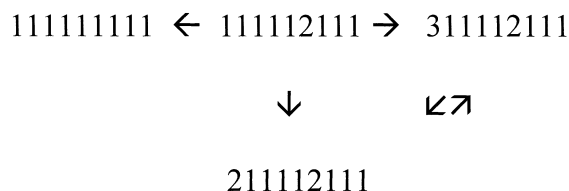


Figure 4 Relationship of the *F. oxysporum* f.sp. *canariensis* haplotypes defined by single-copy RFLPs.

haplotype has a similar, one-step relationship to all other haplotypes. For this reason, it is suspected that isolates from the Canary Islands may represent the ancestral form of *F. oxysporum* f.sp. *canariensis*. This would be consistent with the idea that the pathogen arose from the centre of origin of the host species. Additional information from other polymorphic single-copy loci could be informative for supporting or refuting this hypothesis.

According to mitochondrial haplotypes and single-copy RFLPs, *F. oxysporum* f.sp. *canariensis* appears to be more genetically diverse than *F. oxysporum* f.sp. *albedinis*, the cause of Bayoud disease on *Phoenix dactylifera*. Previously it was found that a collection of isolates spanning the entire geographic range of *F. oxysporum* f.sp. *albedinis* all belonged to a single VCG, and had identical mitochondrial DNA RFLP patterns and RAPD profiles (Tantaoui *et al.*, 1996). Only DNA fingerprinting using the transposable element *FotI* from *F. oxysporum* was able to differentiate the isolates, yielding 23 DNA fingerprint patterns among the 44 isolates examined. Conversely, *F. oxysporum* f.sp. *canariensis* appears less diverse than f.sp. *cubense*, which has been shown to have multiple evolutionary origins. *F. oxysporum* f.sp. *cubense* has at least 16 VCGs with multiple mtDNA haplotypes and at least 10 widely divergent clonal lineages (Koenig *et al.*, 1997).

Genetic distance data derived from pEY10 fingerprints yielded 32 different fingerprints for 57 isolates of *F. oxysporum* f.sp. *canariensis*. The fingerprint patterns are consistent with the idea that pEY10 identifies clonally derived isolates. All isolates with identical pEY10 fingerprint patterns also have the same mitochondrial and single-copy multilocus haplotype. These isolates almost certainly were clones and were often isolated from the same geographical region. Such clones were identified in isolates from California, Florida, Japan, Sicily, France and the Canary Islands (Table 1). On the other hand, DNA fingerprint patterns were sometimes shared by isolates from different geographic locations, suggesting the dispersal of clones. For example, isolates 95-513, 95-514C, 95-913, 95-1000 to 95-1004, 95-1006 and 95-1007 all have the DNA fingerprint profile number 13, yet were isolated from either Manatee County or Lake County, Florida. These counties are separated in distance by about 80 km.

Genetic distance data indicate that isolates from Florida are, in many cases, more closely related to isolates from other parts of the world than they are to

each other. For example, Florida isolate 95-1714 (fingerprint pattern 14 in Fig. 2) is most closely related to isolate 70-MercIV (fingerprint pattern 6 in Fig. 2) from France (distance = 0.03), while it is separated from Florida isolates in fingerprint pattern 13 (Fig. 2) by a genetic distance of 0.284. In another example, isolate 94-861 (fingerprint pattern 11) from Hillsborough County, Florida is separated from California isolates 96-08, 96-13 and 96-14 (fingerprint pattern 2) by a distance of 0.059. On the other hand, the distance between 94-861 and the Florida isolates in fingerprint pattern 13 is 0.284. In these instances, the isolates from Florida are more closely related to isolates from distant areas than to each other. As the presence of the fungus in Florida was not documented prior to 1994, it is unlikely that the Florida isolates have evolved these levels of differences within the state; these observations appear to support the idea that *F. oxysporum* f.sp. *canariensis* arrived in the state through multiple, independent introductions. Transport of contaminated vegetative or seed palm material would probably be a method of introduction to Florida.

In summary, the data presented here support the idea that *F. oxysporum* f.sp. *canariensis* is derived from a single lineage. However, the pathogen has diversified in various regions of the world and has probably also been introduced into new areas through international trade. Recently developed diagnostic systems for the pathogen (Plyler *et al.*, 1999) will aid in the early detection of the fungus on live plants, and facilitate efforts for exclusion of the pathogen and production of pathogen-free transplant materials.

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